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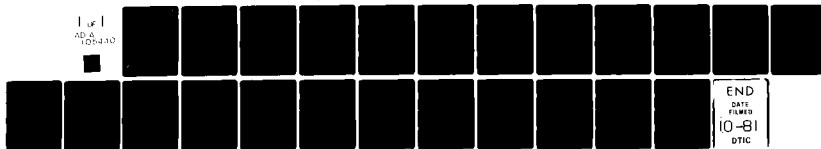
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In this first year we have assembled all of the resources and skills required for this project and have completed many preliminary and preparative studies which will provide the reagents and information which are required for its fulfillment. In particular, we have established a P-2 Biohazard experimental facility for virus propagation and experimentation. We are producing several monoclonal antibodies needed for this study and are testing new methods to favour the production of the desired antibody specificity.

➤ We have developed an experimental system to study Herpes Virus (HSV) immunity in man. Individuals with primary and with recurrent disease have been recruited to our donor panel and, using their cryopreserved lymphocytes, immune responses have been generated in tissue culture to this pathogenic virus and to its components. From these responses T cell lines have been produced and propagated in vitro. These lines exhibit the properties of a pool of T cell clones having immunity to HSV. In addition numerous clones of T lymphocytes (over 100) derived from single precursor cells have been produced and expanded to large numbers for study using these new tissue culture methods.

Large scale production of virus and of selected antigenic components has been completed and tested. These materials are being used as antigens in various immunizations, in assays of T-cell immunity and in serological tests.

In the mouse model we are analyzing the ability of immune T cells to protect normal mice from the lethal HSV infection. We have succeeded to produce T cell lines and clones of virus-immune T lymphocytes and are now testing their specificity, phenotype and function.

The production of monoclonal antibodies to human MHC antigens, needed for analysis of the functional properties of T cell clones, is well advanced. Immunizations to produce virus-specific monoclonal reagents are now in progress.

Thus, this laboratory has begun the large scale production of T cell clones and monoclonal antibodies which are required for detailed functional studies and for future applications in immunodiagnosis, immunotherapy and defensive immunization.

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OFFICE OF NAVAL RESEARCH
 Contract N000-14-80-K-0909
 Task No. NR 202-179
 Technical Report No. 1

IMMUNOTECHNOLOGY:
 PREPARATION OF IMMUNOTHERAPEUTIC REAGENTS AND
 DEVELOPMENT OF IMMUNOPHARMACOLOGIC VACCINES

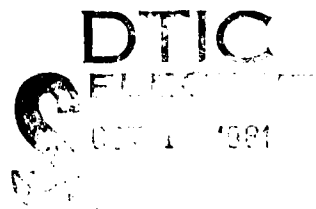
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September 1981

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A. ABSTRACT

The objective of this contract is to explore the potential application of several recent and expanding discoveries in immunology to the problems of diagnosis and therapy of human disease. These recent discoveries include 1. the production of monoclonal antibodies, 2. the cloning of immune T-lymphocytes, and 3. the manipulation of immunity using anti-idiotypic antibodies.

This contract was established to design and conduct studies, using a human pathogen coupled with experimental models in vitro and in animals, to demonstrate the usefulness of these new technologies for 1. the rapid diagnosis of diseases or agents and to test their 2. potential to confer solid immunity to individuals who are at risk.

In this first year we have assembled all of the resources and skills required for this project and have completed many preliminary and preparative studies which will provide the reagents and information which are required for its fulfillment. In particular, we have established a P-2 Biohazard experimental facility for virus propagation and experimentation. We are producing several monoclonal antibodies needed for this study and are testing new methods to favour the production of the desired antibody specificity.

We have developed an experimental system to study Herpes Simplex Virus (HSV) immunity in man. Individuals with primary and with recurrent disease have been recruited to our donor panel and, using their cryopreserved lymphocytes, immune responses have been generated in tissue culture to this pathogenic virus and to its components. From these responses T cell lines have been produced and propagated in vitro. These lines exhibit the properties of a pool of T cell clones having immunity to HSV. In addition numerous clones of T lymphocytes (over 100) derived from single precursor cells have been produced and expanded to large numbers for study using these new tissue culture methods.

Large scale production of virus and of selected antigenic components has been completed and tested. These materials are being used as antigens in various immunizations, in assays of T-cell immunity and in serological tests.

In the mouse model we are analyzing the ability of immune T cells to protect normal mice from lethal HSV infection. We have succeeded to produce T cell lines and clones of virus-immune T lymphocytes and are now testing their specificity, phenotype and function.

The production of monoclonal antibodies to human MHC antigens, needed for analysis of the functional properties of T cell clones, is well advanced. Immunizations to produce virus-specific monoclonal reagents are now in progress.

Thus, this laboratory has begun the large scale production of T cell clones and monoclonal antibodies which are required for detailed functional studies and for future applications in immunodiagnosis, immunotherapy and defensive immunization.

B. INTRODUCTION

The following technical report summarizes our progress in the first eleven months on ONR Contract N-14-80-K-0909 entitled: "Immunotechnology: Preparation of Immunotherapeutic Reagents and Development of Immunopharmacologic Vaccines." This project was initiated in October 1980 to adapt some new and important technological advances in Immunology for use in the detection, diagnosis, therapy and immunization of individuals to biohazardous infectious agents.

C. BACKGROUND

Several new immunological technologies have emerged recently which are likely to have important impact on approaches to prophylaxis, diagnosis and therapy of infectious diseases. These new methods permit the production of monoclonal antibodies and monoclonal T cells. Apart from the obvious benefit of increased quantities of antibodies and cells with which researchers may probe the complexities of the immune response and of antigens, it now becomes possible in practice to test strategies of providing immunity by transfer of immune cells or antibodies, or in more sophisticated approaches, to produce immunity by stimulating cells to become immune using anti-receptor (idiotype) antibodies in vivo and in vitro.

It has been long recognized that immunity mediated by T cells is crucial in protection and recovery from viral diseases. T cell immunity is considered to be mediated by 1. helper T cells (TH) which are responsible for aiding B cells to produce antibody and for producing delayed type hypersensitivity (DTH) and by 2. cytotoxic T cells (Tcx) which kill virus infected cells before they produce new mature viruses. In most cases of virus disease antibody plays little part. It was therefore not surprising that the transfer of T cells

immune to Influenza could protect normal mice from the disease, but it was of particular interest that the T cells which mediate killer (Tcx) function were critical for this effect (1).

In man, T mediated cellular immunity is vital in controlling viral infections but the role of the different T cell types is not known and as these cells could not be grown in culture understanding has progressed slowly.

Very recently it was recognized that supernatants from mitogen stimulated lymphocyte cultures can maintain the growth of T-lymphocytes in vitro (2). This effect is mediated by a hormone termed T cell growth factor (TCGF) or (IL-2) Interleukin 2. TCGF has allowed the expansion and maintenance of functional antigen-specific T cell subpopulations in long-term culture (3). Thus, in the mouse it has been possible to prepare antigen-reactive clones of T-cells that recognize specific alloantigenic determinants (4), particulate antigens such as SRBC or soluble protein antigens (5).

In man, most early studies followed the proliferation of PBL to antigens such as PPD to analyze cell interactions (6). More recently long term cultures of human T-lymphocytes in TCGF have been reported, including lines and clones specific for antigens (7).

However no reports exist on the production of human T cell clones with specificity for infectious agents such as viruses. Consequently we have no information on the properties of the antigens which are recognized by these cells, the genetics of antigen presentation or the properties and functions of the immune cells.

D. RESEARCH DESIGN AND PLAN

The approaches to this project are listed below.

1. Establishment of a laboratory area suited for research using biohazardous pathogens, and formulate guidelines and work routines for laboratory personnel.
2. Organize a panel of reliable human volunteers and paid blood donors including individuals with Herpes Simplex Virus Types I and II infections and institute a procedure for peripheral blood lymphocyte separation and cryopreservation of the cells.
3. Production of virus batches, subviral antigenic components and inactivated virus preparations, anti-viral antisera and development of serological assays for monoclonal antibodies.
4. Begin the production of monoclonal antibodies to strong antigens to establish this system and to obtain anti-MHC antibodies for analysis of viral antigen presentation. Prepare anti-HSV monoclonal antibodies, for a similar analysis, for adoptive protection studies and for anti-idiotypic immunizations. Adapt the ELISA test using fixed cell monolayers as antigenic targets for the detection of monoclonal antibodies.
5. Develop proliferation assays to HSV antigens using human peripheral blood lymphocytes as responding cells.
6. Develop methodology, using the proliferation assay as a starting point, for the production of human T lymphocyte lines and then of T cell clones specifically reactive to HSV antigens.

7. Characterize these lines and clones for 1. cell surface phenotype, 2. fine antigen specificity, 3. genetic restriction and function including helper function and killer activity.
8. Prepare (anti-idiotypic) antibody to the antigen receptors on the T cell clones and use this reagent to immunize T cells from non-immune individuals to HSV antigens in vitro.
9. Test the lines and clones immunized in vitro (with anti-receptor antibody) for phenotype, antigen specificity genetic restriction and function to show their likeness to cells immunized in vivo.
10. Parallel studies of proliferation in mice are to be performed using mixed lymphoid cells from animals immunized in vivo with various preparations of HSV which produce the most effective immunity. These immune cells are then assayed in vivo (protection experiments) and in vitro (antigen-induced proliferation).
11. Lines and clones of mouse T-lymphocytes immune to HSV will be grown in tissue culture characterized for surface phenotype, fine antigen specificity, genetic restriction and function in vitro.
12. Adoptive protection assays (protection from death) will be performed on normal infected mice to demonstrate the functional activity of in vitro grown immune cells.
13. Anti-idiotypic antisera produced to mouse monoclonal antibodies will be tested for ability to stimulate T and B cells in vivo to provide protective immunity in the intact animal and also tested in vitro to sensitize cells which can, on adoptive transfer, produce solid immunity in vivo.
14. Initiate ties with other Navy contract facilities where expertise in cellular immunology would complement their research efforts.

E. RESULTS

1.0 Biohazard Laboratory Established and Personnel Trained

Since the research plan requires the use of virally transformed cell lines and several viruses with known pathogenic and possible oncogenic potential in man, it was necessary to design and construct a controlled-access P-2 laboratory. This involved 1. the construction of an enclosed laboratory, 2. installation of special venting fans to assure adequate negative air pressure, 3. installation of two Class 2B Biological Safety Cabinets vented after filtration to the exterior, 4. publication and display of laboratory rules and regulations for all research personnel derived from guidelines published by the National Cancer Institute, and 5. the installation of other major equipment items including work benches, incubators, centrifuges and other heavy items.

New personnel were recruited and trained in the technology required by this laboratory. This included 1. the culture and inactivation of viruses, and methodology of virus PFU assays, cryopreservations of cells, culture of human cells and cell lines, collection, separation and handling of human blood cells and plasma, cell proliferation assays using radioactive methods, production of growth factors required for the propagation of human T cell lines and clones, methodology for anti-virus cell-mediated cytotoxic assays, methodology for the production of monoclonal antibodies from mice and for the assay of antibodies by ELISA solid-phase colorimetric methods.

2.0 Human Immune Responses to Herpes Simplex Virus Types I and II
(HSV I and II)

2.1 Donor Recruitment, Cell Cryopreservation and HLA Typing of
the Panel

We have assembled a group of volunteer and paid blood donors, some of whom have been selected on the basis of having a history of recurrent HSV infections. In addition, donors experiencing initial infections of HSV are being recruited through the cooperation of several local health centers and the Washington Chapter of HELP, a Herpesvirus support group.

Donor blood is separated on Ficoll-Hypaque according to standard procedures and the leukocytes are aliquotted and frozen following procedures developed in this laboratory in FCS and DMSO and stored in vapor phase N₂. Computerized records are kept of cell inventories. In addition donor plasma is stored frozen for anti-viral antibody titers and individuals are typed for ABO and Rh antigens.

For the cell cultures, human A⁺ plasma is required and a panel of paid donors, recruited from the medical school, is used. All reagents are pre-screened for their ability to sustain human cell cultures and mixed leukocyte responses in vitro with standardized reagents used as controls.

The cell donor panel is typed for human histocompatibility antigens. Dr. A.H. Johnson determines the HLA-A,B,C phenotypes and Dr. R. Hartzman performs the Dw assays by PLT and MLR typing. In this way we select donors of known tissue types which can permit further detailed analysis of the genetically controlled events involved in immune activation.

2.2 Production of Virus and Viral Antigens

It is unknown at present which of the many virus components constitute the dominant antigens against which the immune system responds. To explore this problem in HSV immunity we have obtained several virus isolates of HSV I and II and have begun the production of subviral antigen extracts.

The replication of HSV involves three major waves of virus polypeptide synthesis (8) immediate early, early and late groups. It is unknown which of these groups of antigens is most important in immunity. To test for a role of each antigen in human immunity we have produced immediate early antigens using the cycloheximide-Actinomycin D methods (9), early antigens using the drug, phosphonoacetic acid (10), and late antigens using cell monolayers with completed virus lytic cycles (CPE). In addition we have prepared sucrose density gradient-purified virions of HSV to examine the antigenic properties of virions lacking cell associated, virus-coded antigens.

In these procedures monolayers of BHK or VERO cells were infected with a seed stock of HSV (grown at MOI of 0.1 for 24h) at an MOI of 1.0 (or 20.0 for drug blocked cultures). Following adsorption of virus for 1h at 37°C in serum-free conditions drugs were introduced and cell monolayers harvested at 24 hours by freezing and sonication.

As will be shown below these laboratory antigen preparations are highly effective in stimulating proliferative responses in HSV-immune lymphocytes.

Production of virus in both VERO cells and BHK cells was necessary in order to control for contributions to antigenicity by the host cells. These methods have yielded large quantities of virus (5×10^8 pfu per ml) which have been quantitated using a virus plaque assay on VERO cell monolayers.

2.3 In vitro proliferation responses

Peripheral blood leukocytes from our frozen donor panel have been thawed and placed into culture with various mitogens and antigens. Responses were examined daily for nine days by adding ^3H -thymidine and quantitating cell proliferation by scintillation spectroscopy. In preliminary studies it was found that primary responses to antigens as Influenza A(A/Texas), and Tetanus toxoid showed peaks on days 6-7. Similarly responses to different HSV antigen preparations showed peaks after one week. Secondary responses were induced in vitro by replacing one-half of the culture supernatants with fresh medium containing irradiated PBL (a source of antigen presenting cells) and HSV antigen. These responses peaked at days 3-5. In addition to being more rapid and stronger the responses were now highly specific for the antigen which induced the primary response.

In this way we may begin to study human secondary in vitro responses. In Table I, data representative of these proliferation responses are shown. The

TABLE I

Proliferative response of PBL from infected and normal human volunteers to HSV antigen preparations

Donor	Proliferative response (CMP) to:				
	MEDIUM	VERO	VERO HSV-1	VERO HSV-II	PHA
LAM	115	153	13,299	7,988	124,379
SMA	507	425	5,235	4,399	144,519
LNA	122	151	3,435	1,181	141,322
BRO	791	809	8,621	24,504	153,526

PBL responses were determined on day 6 after antigen stimulation. Infected cell (VERO) monolayers were harvested, sonicated and used as antigen.

results were obtained from an experiment in which PBL from five donors were cultured with medium alone, HSV antigens, control VERO cell lysate or the T-cell mitogen PHA. The results show clearly that cells from the four individuals with HSV disease proliferate strongly in response to HSV antigens but not to the control antigen. In addition cells from LNA and LAM (who have only Type I virus) react strongly to antigens of Type II. This is the first observation that cellular responses are highly cross-reactive between the HSV virus types. It is of some interest that individuals with immunity to Type I virus can become severely ill with primary Type II disease. This suggests that those antigens which are shared or which cross-react among types I and II are not currently involved in providing protective immunity.

2.4 Production of T cell lines specific for HSV-I

There are many advantages in the study of antigen specific lines and clones of T lymphocytes as compared with whole populations of PBL cells. These include 1. the uniform antigen specificity of the line or clone since, when properly produced, it is fully specific to the stimulating antigen and reacts with no other; 2. the response kinetics are more rapid since the cells are previously primed in vitro and the magnitude is greater since virtually all the T cells in the line or clone are potential responders, while only a small fraction of those in PBL are specific for HSV; 3. there is no problem of allospecificity, that is, the capacity of PBL to respond to PBL's from another individual in an MLC response. Eliminating the MLC allows the experiment to test the genetic requirements for antigen presentation simply, by changing the donor source of the irradiated PBL; 4. lines and clones can be expanded to vast numbers and then frozen. Upon thawing they respond to antigen in the presence of PBL thus permitting excellent reproducibility; 5. clones of cells

are not subject to cell-cell regulation (for example by suppressor cells) that may occur in responses of mixed cell populations. Thus the responses of single clones can be studied either unmodulated or (by mixing with PBL or other clones) in conditions where cell network controls operate.

However, to prepare lines and clones of T cells the hormone TCGF (or IL-2) is required. The background technology for the production of human TCGF was reproduced fully including 1. the preparation of pools of screened A⁺ plasma; 2. expansion of a TCGF dependent T cell line (CTC 4), which is PHA insensitive, for assay of TCGF activity; and 3. preparation of numerous litre-size lots of TCGF and screening the activities.

HSV specific T cell lines were induced to HSV antigens by harvesting primary response cultures and growing the cells in tissue culture medium containing TCGF. Expansion in TCGF with occasional admixtures of pooled irradiated feeder cells continued until adequate cell numbers were obtained, eg. 30×10^6 . At this point (1-2 weeks of growth) the line was frozen in N₂.

The response of one such line induced to HSV antigens is shown in Table II. It is apparent that the line responds well to HSV antigens and virions but

TABLE II
Response of a T Cell Line Induced to HSV Antigens
Upon Stimulation With Various Antigens

	Day of Test	Proliferative response (CPM) to						
		Medium	VERO	VERO HSV	VIRION HSV	Flu A	Tet T	PHA
Exp. 1	3	37	13	1,395	3,461	19	25	23,049
	5	248	397	5,757	5,622	182	105	111,704
Exp. 2	3	169	348	20,726	14,456	349	770	30,140

Antigen doses of Tet. T and Flu A were chosen which produced optimal proliferative responses of PBL cells. These PBL responses were larger than those to HSV.

virtually not at all to tetanus toxoid or Influenza A. The latter antigens, it should be noted, are more effective than HSV to stimulate PBL primary responses, underlining the dramatic antigen-driven selection the cell population underwent in becoming a cell line.

2.5 Production of T cell clones specific for HSV-I.

T cell clones have all of the advantages of T cell lines for analysis of HSV responses as well as an additional attribute. Since a clone is derived from a single precursor cell, the progeny population is monospecific - that is, only a single antigenic site is recognized and antigen presentation requirements for all the cells are identical. This allows for fine dissection of the T cell response which is impossible by any other approach.

T cell clones were induced from human primary in vitro responses to HSV antigens. Cells taken from day 5 primary responses were plated in Terasaki trays at a concentration of 1 cell per 3 wells in the presence of irradiated syngeneic feeder cells HSV antigens and TCGF. After six days of culture, wells containing growing cells (approximately 5% of wells) were expanded in larger vessels using similar conditions until approximately 5 to 10 x 10⁶ cells had been grown and frozen.

Prior to freezing, the clones were tested for reactivity to antigen in a three day culture in the presence of antigen and antigen-presenting PBL (irradiated). As can be seen in Table III in response to HSV antigens excellent proliferation of the T cells is seen. This response is specific

TABLE III
Responses of T Lymphocyte Clones Induced to HSV-I

CLONE	Proliferative response (CPM + SD) to:							
	+PBL (irr) +VERO HSV (SD)		+PBL (irr) +VERO (SD)		MEDIUM (SD)		TCGF (SD)	
88	16,403	3,729	63	24	21	1	2,800	195
36	15,096	1,226	49	9	45	1	1,217	440
65	13,401	3,388	17	4	23	8	7,727	596
12	2,506	434	23	4	16	0	14,115	2,503

Responses of four clones representative of over 50 isolated are shown.

for the four clones shown with responses to specific antigen from 100 to over 700 fold above responses to the irrelevant antigen, VERO. Other clones (not shown) which displayed responses to VERO antigens as well as clones with no response to either antigen are excluded from further study.

To date over 50 clones have been selected for future detailed study on the basis of excellent responses to HSV antigens and no responses to other antigens tested.

3.0 Animal Model for HSV Infection

It would be very useful to carry out parallel studies in mice, which are highly susceptible to HSV disease, since immunizations with virus and subviral material would be possible and also, it would be possible to test T cell lines and clones for activity in vivo. Work in this area is now developing since our new animal holding facilities, required for the handling of diseased mice shedding large amounts HSV Type I and II, has become available.

Following footpad immunization cells, from the draining lymph nodes show proliferative responses to HSV antigens, however as these responses are weak we are continuing to develop this assay and are also attempting to expand immune T cells in irradiated mice. Cells from such irradiated hosts were expanded in TCGF as a line and also were cloned successfully. Initial studies with the line shows excellent specificity for HSV.

4.0 Monoclonal Antibody Production

An important new technology is the advent of monoclonal antibody production from the somatic fusion of malignant tumor cells with antibody producing cells. Our laboratory contributed to this technology (11) and we now plan to explore the application of this technology to diagnosis immune therapy and vaccination against pathogenic agents. We also plan to raise monoclonal antibodies to cell surface antigens in order to characterize the phenotypes of responding cells, and to analyze the mechanism of virus antigen presentation to T cells. Using mice immunized with human T cell clones we plan to raise conventional and monoclonal anti-idiotypic antisera which may be useful in priming normal human lymphoid cells in vitro to HSV antigens.

Work in this area began with the production of monoclonal antibodies to human T and B cells in order to generate anti DR, anti beta 2 microglobulin and anti-HLA antibodies. Screening the fusions on glutaraldehyde fixed monolayers of B lymphoblastoid lines, T cell leukaemic lines and on human platelets, we have produced a number of strongly binding candidate reagents. These clones are being expanded for inoculation into mice to generate sufficient antibody for immunoprecipitation of cell surface antigen to confirm their specificity.

Work has already begun in an effort to raise anti-idiotypic antibodies to several of our human T cell clones by hyperimmunization with these cells.

F. PROPOSAL FOR CONTINUATION OF CONTRACT AND WORK PLAN

1. Production of Lines and Clones to HSV Components.

We plan to extend the studies on HSV specific T cell lines and T cell clones as follows: production of lines and clones a) from several individuals, b) to IE, E and late viral antigens, c) to heterotypic HSV antigens. We can then study the functional properties of these lines (eg. cytotoxic T cells specific to HSV) to determine more precisely which antigens are salient to the response. We will also vary culture conditions to identify the parameters which control the development of cytotoxic and helper-type T clones.

2. Production of Genetic Variants of HSV Antigens and Purified Viral Glycoproteins and Polypeptides

We are planning to use the HSV intertypic recombinant viruses now available and others being generated from cloned DNA fragments to map the antigens which stimulate HSV T cell responses on the HSV genome. In addition, ongoing collaboration with laboratories at the Frederick Cancer Center will provide us with immunoaffinity purified HSV glycoproteins to identify the precise molecular antigen to which each clone is sensitized.

3. Analysis of HSV-Specific T Cell Clones: Cell Phenotype MHC Restriction, Function and Properties.

With our ORTHO Diagnostics Cell Sorter we have begun to phenotype the T clones with respect of T cell markers including the subset markers of OX40, and 8 of helper and cytotoxic cells. Antigen recognition by T cells involves presentation of antigen on HLA structures. Since Th cells are considered to recognize antigen with DR molecules while Tcx cells require HLA A or B antigen recognition, the respective responses can be blocked by monoclonal antibodies to the relevant HLA antigen. An alternate approach will include using our panel of HLA-typed cells where responses occur only when antigen is presented to the T cell clone by donor cells which share the HLA region required by the T cells.

Effector functions of T cells include DTH inflammation, helper effects for antibody production and (Tcx) the killing of infected cells. We will induce antibody responses to HSV in vitro in order to assess Th activity and use ⁵¹Cr-release assays of HSV infected target cells to assay Tcx activity. In collaboration with colleagues plan to examine clones for the release of lymphokines including TCGF, MIF, TRF, THF and interferon.

4. Extension of Cell Fusion Technology

This laboratory is working with a new human B cell myeloma suited for preparing clones of cells which secrete human antibody. It is desirable to induce antibody responses in vitro from human cells for this purpose and our study will include responses to HSV. In addition we shall study the variables which control the in vitro antibody response in order to obtain suitable antibody-secreting cells suitable for fusion.

5. Development of the Animal Model

Efforts will continue to produce strong proliferative responses to HSV in lymph node cultures of immunized mice. From these bulk cell cultures clones of HSV immune cells will be grown, tested for HSV reactivity and reinoculated into mice to protect the animals from lethal HSV disease. In parallel, experiments on adoptive transfer of immunity using monoclonal antibodies will be developed. These experiments should provide the groundwork for anti-idiotypic immunization in vivo which is to follow.

G. PUBLICATIONS

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